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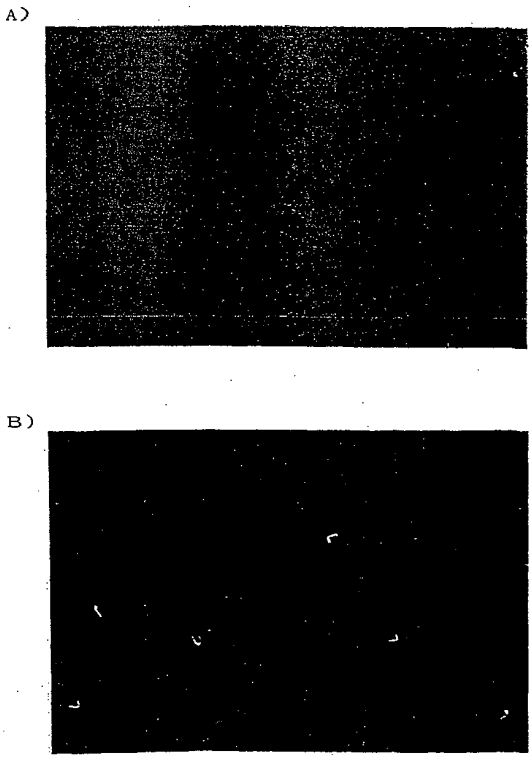
(54) ANTIBODY AGAINST RAT POSTACROSOME REACTION SPERM AND UTILIZATION THEREOF

(57) An antibody biding specifically to rat's acrosome reacted sperm is produced and hybridomas (FARS-91 and FARS-92 strains) capable of stably proliferating are obtained by fusing mouse spleen cells having a high antibody titer against rat's acrosome reacted sperm with mouse-origin myeloma cells and screening fused cells reacting strongly with rat's acrosome reacted sperm. From these hybridomas, monoclonal antibodies selectively binding to rat's acrosome reacted sperm can be obtained. Thus, a diagnostic method for evaluating fertility of rat's spermatozoa is presented.



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Description

Technical Field

[0001] The present invention relates to monoclonal antibodies selectively binding to rat's acrosome reacted sperm, hybridomas producing said monoclonal antibodies, and a method for evaluating fertility of rat's spermatozoa using said monoclonal antibodies. The invention also relates to a method for screening materials effecting on the rat's fertility, characterized in using said method for evaluating fertility.

Background Art

[0002] It is now obligated to do reproductive and developmental toxicity studies in studies relating to safety of a drug on the occasion of an application of manufacturing approval for the drug. The reproductive and developmental toxicity studies mean animal experiments that are conducted to obtain information on whether the application of a drug to living bodies could possibly induce some adverse effect in the course of reproduction and development. The experimental results can be extrapolated to humans and utilized in evaluation of safety (risk) of the drug to reproduction and development in humans. A guideline for reproductive and developmental toxicity studies promulgated on 1997 was revised and enacted in conformity with the ICH guideline (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; at the international conference for harmonization of regulation of pharmaceuticals in three areas involving Japan, USA and EU) which was advised on 1994. In this guideline, it is described that histopathological examinations of reproductive organs and a sperm analysis in repeated-dose toxicity studies should be made relative to evaluation of male fertility in studies relating to fertility and early embryonic development to implantation.

[0003] To perform the histopathological examination for male reproductive organs, however, skillful technique is required, and there is sometimes a case given an effect to spermatozoa which could not be detected by means of the histopathological examination. In order to correctly evaluate fertility and precisely examine an effect of a drug on spermatozoa, it is necessary to perform the fertility examination of spermatozoa themselves as well as the histopathological examination.

[0004] In the current reproductive and developmental toxicity studies, there are no examinations for concentration, motility, morphology of spermatozoa and the like that have been performed in a human sperm analysis. Accordingly, an investigation for establishing a method for evaluation of spermatozoa motility is now being continued. This means that a method for evaluation of fertility has not yet been established.

[0005] As for examinations of fertility of human spermatozoa, a hamster test, conserved zona pellucida passage test, and triple-stain test and the like have been developed. The hamster test is a method for evaluation of fertility, in which are utilized properties of spermatozoa, that is, the only spermatozoa which have capacitation and in which the acrosome reaction have been completed, can enter the zona-free hamster eggs. The conserved zona pellucida passage test is a method of utilizing properties of spermatozoa, that is, only spermatozoa which have capacitation, in which the acrosome reaction have been completed and in which the motility is retained sufficiently, can pass through the conserved pellucid zone. The triple-stain method is a method for distinguishing dead spermatozoa, live spermatozoa and spermatozoa of acrosome intact by means of staining them by a combination of three types of staining methods.

[0006] When the above-mentioned test that is so far known for human spermatozoa is applied to the reproductive and developmental toxicity studies, it is necessary to collect eggs to eliminate the zona pellucida, or to permit maturation of the collected immature eggs in vitro and preserve them in a particular salt solution at a high concentration to form the zona pellucida. These procedures, however, require skillful technique and troublesome operation as well as expensive equipment for experiment. When influence of chemical materials such as drugs or environmental hormones on fertility of spermatozoa is examined, in the current reproductive and developmental toxicity studies the decision whether or not to be fertilized on experimental animal is not determined from their appearance as far as the fetus has grown up in some degree after mating. This necessitates time and cost. Even though many points such as a number of spermatozoon, mobility and morphology, which appear to have somewhat influence on fertilization, are examined, it is impossible to determine the exact fertility as far as the presence of acrosome reaction as an essential factor for fertilization can't be confirmed.

Disclosure of Invention

(Problems that the Invention is to Solve)

[0007] It is now necessitated, in the current reproductive and developmental toxicity studies, to develop an accurate and conveniently operable method for evaluating fertility of spermatozoa, by which the test completes within a short period of time. It is also necessary that the test results in the above method can be extrapolated to humans.

(Means for Solving the Problems)

[0008] The present inventors paid attention to utilize an antigen-antibody reaction in order to solve the above problems.

[0009] And, the present inventors have succeeded in

preparing monoclonal antibodies specific to non-human animals spermatozoa posterior to acrosome reaction as well as hybridoma producing said monoclonal antibodies. At the same time, using these monoclonal antibodies, they established a method for evaluating fertility of spermatozoa, wherein the test results can be extrapolated to humans, and they also prepared a composition used in determination of fertility. Moreover, they also established a method for screening materials effecting on fertility.

[0010] According to the invention, it is possible to finish the tests within a short period of time and to evaluate fertility of spermatozoa accurately in a convenient operation.

[0011] The principle of evaluation of fertility in the invention is as follows. The spermatozoa just after ejaculation of mammals have no ability of fusion (fertilization) with an egg. First, adsorptive materials such as glycoproteins and glycolipids contained in epididymis secretion or seminal fluid, which cover the surface of protoplasmic membrane of the spermatozoa to protect the spermatozoa in female reproductive organs , are removed or denatured. This physiological alteration is called "capacitation". Subsequently, the spermatozoa reaching the ampulla of tubue uterinae, which is a spot for fertilization, make an approach to an egg and cause "acrosome reaction". The acrosome reaction means a phenomenon in which the outermost protoplasmic membrane of spermatozoa fuses to the acrosomal outer membrane enveloping the outside of acrosome to open a small pore, through which an acrosomal enzyme is released from the acrosome. Only the spermatozoa that have caused the acrosome reaction can fuse to the egg (fertilization) through dissolution of oval ambient pellucid zone by the acrosornal enzyme. Accordingly, occurrence of the acrosome reaction is an essential condition for fertilization, and confirmation of the occurrence of the acrosome reaction is a direct method for evaluating fertility of spermatozoa. The method of the invention, accordingly, can be used in evaluation of fertility of spermatozoa conducted in rats for the purpose of the reproductive and developmental toxicity studies and the like, on the assumption that the obtained test results can be extrapolated to humans.

[0012] In this connection, in order to carry out the evaluation of fertility used in extrapolation to humans , it is necessary to choose experimental animals whose physiological state is very similar to that of humans. Until now, monoclonal antibodies to non-human animal acrosome reacted sperm have been reported on mice (Masaru Okabe et al., Journal of Reproductive Immunology, 11(2), 91-100, 1987), though as experimental animals used in extrapolation to humans, rats whose general metabolic way is well known have widely been used.

[0013] Therefore, monoclonal antibodies by which the occurrence of acrosome reaction of rat's spermatozoa can be distinguished were produced using rats, animal species, which are most experienced in experimental reproduction and development and of which the physiological state is very similar to that of humans.

[0014] At present, as for pharmaceuticals inhibiting the acrosome reaction of human spermatozoa, bicuculline (Calogero A.E. et al; Fertility and Sterility, 71 (5), 930-936, 1999), wortmannin (Fisher H.M. et al ; Molecular Human Reproduction, 4(9), 849-855, 1998), cysteamine (Sengoku K. et al; Journal of Andrology, 19(1), 37-49, 1998), anantin (Rotem R. et al; American Journal of Physiology, 274(2 Pt 1), E218-223, 1998), chlordane and endosulfan (Turner K.O. et al; Journal of Andrology, 18(6), 571-575, 1997), etc., have been reported. In the future, monoclonal antibodies to acrosome reacted sperm will be useful in order to investigate an effect of drugs on the acrosome reaction of spermatozoa in the reproductive and developmental toxicity studies.

Brief Description of Drawings

[0015]

Fig. 1 is a photomicrograph showing reactivity of the antibody EARS-91 to acrosome reacted sperm (spermatozoa as control treated with A23187).

A) Reactivity of the antibody FARS-91 with the control spermatozoa (bright field; magnification of 100)
B) Reactivity of the antibody FARS-91 with the control spermatozoa (FITC excitation; magnification of 100)
A and B shows the same field of vision.

Fig. 2 is a photomicrograph showing reactivity of the antibody FARS-91 to acrosome reacted sperm (spermatozoa treated with A23187).

A) Reactivity of the antibody FARS-91 with spermatozoa treated with A23187 (bright field; magnification of 100)
B) Reactivity of the antibody FARS-91 with spermatozoa treated with A23187 (FITC excitation; magnification of 100)
A and B shows the same field of vision.

Fig. 3 is a photomicrograph showing reactivity of the antibody FARS-92 to acrosome reacted sperm (spermatozoa as control treated with A23187) .

A) Reactivity of the antibody FARS-92 with the control spermatozoa (bright field; magnification of 100)
B) Reactivity of the antibody FARS-92 with the control. spermatozoa (FITC excitation; magnification of 100)
A and B shows the same field of vision.

Fig. 4 is a photomicrograph showing reactivity of the

antibody EARS-92 to acrosome reacted sperm (spermatozoa treated with A23187).

A) Reactivity of the antibody FARS-92 with spermatozoa treated with A23187 (bright field; magnification of 100)
B) Reactivity of the antibody FARS-92 with spermatozoa treated with A23187 (FITC excitation; magnification of 100) A and B shows the same field of vision.

Fig. 5 is a photomicrograph showing the frozen and thawed spermatozoa observed by FITC-PSA staining.

A) Fresh spermatozoa (positive; magnification of 400)
B) Frozen and thawed spermatozoa (negative; magnification of 400)

Fig. 6 is a photomicrograph showing reactivity of the antibody FARS-91 with the frozen and thawed spermatozoa.

A) Reactivity of the antibody FARS-91 with fresh spermatozoa (negative; magnification of 200)
B) Reactivity of the antibody FARS-91 with the frozen and thawed spermatozoa (positive; magnification of 200)

Fig. 7 is a photomicrograph showing reactivity of the antibody FARS-92 with the frozen and thawed spermatozoa.

A) Reactivity of the antibody FARS-92 with fresh spermatozoa (negative; magnification of 200)
B) Reactivity of the antibody EARS-92 with the frozen and thawed spermatozoa (positive; magnification of 200)

Fig. 8 is a photomicrograph showing reactivity of the antibody EARS-91 with the frozen and thawed spermatozoa after treatment with A23187.

A) Reactivity of the antibody FARS-91 with the frozen and thawed spermatozoa after treatment with A23187 (bright field; magnification of 100)
B) Reactivity of the antibody FARS-91 with the frozen and thawed spermatozoa after treatment with A23187 (FITC excitation; magnification of 100)
A and B shows the same field of vision.

Fig. 9 is a photomicrograph showing reactivity of the antibody FARS-92 with the frozen and thawed spermatozoa after treatment with A23187.

A) Reactivity of the antibody EARS-92 with the frozen and thawed spermatozoa after treatment with A23187 (bright field; magnification of 100)
B) Reactivity of the antibody FARS-92 with the frozen and thawed spermatozoa after treatment with A23187 (FITC excitation; magnification of 100)
A and B shows the same field of vision.

Fig. 10 is a photomicrograph showing reactivity of the antibodies FARS-91 and EARS-92 with the frozen and thawed human spermatozoa.

A) Reactivity of the antibody FARS-91 with the frozen and thawed human spermatozoa (FITC excitation; magnification of 200)
B) Reactivity of the antibody FARS-92 with the frozen and thawed human spermatozoa (FITC excitation; magnification of 200)

Best Mode for Carrying Out the Invention

[0016] According to the invention, monoclonal antibodies to rat's acro some reacted sperm and two kinds of hybridomas, FARS-91 and FARS-92, producing said antibodies were obtained. These hybridoma were deposited respectively in the Ministry of International Trade and Industry, National Institute of Bioscience and Human-Technology (NIBH), located in 1-1-3, Higashi, Tsukuba-shi, Ibaragi, 305-8566, Japan, as the accession nos. FERM BP-7401 and FERM BP-7402, on October 13, 1999.

[0017] In producing the above antibodies, first of all rat's fresh spermatozoa which are collected by ejaculation or by removal of the cauda epididymis or seminal duct and which have not led to acrosome reaction (hereinafter referred to as rat's fresh spermatozoa) are provided and induced to acrosorne reaction. Next, the rat's acrosome reacted sperm as antigens are immunized to mammals. The rat's acrosome reacted sperm may be prepared by induction of the acrosome reaction by a chemical procedure, by induction of pseudo acrosome reaction, by induction of the acrosome reaction in culture, or by a combination of these procedures and the like.

[0018] The fresh spermatozoa may be obtained, for example, from the extirpated cauda epididymis or seminal ducts. When the spermatozoa are collected from the cauda epididymis, said cauda epididymis is cleaved at 3 or 4 parts. When the seminal ducts are used, one is cut into 3 to 5 portions. These are then immersed and gently swirled in a culture medium such as TYH medium containing 0.4% BSA or m-KRB medium containing 0.5% BSA, at 30 - 40°C, for example, for 10 minutes, until the spermatozoa migrate. Alternatively, when col-

lected from the cauda epididymis, the spermatozoa may be obtained by cutting a part of seminiferous tubule removed from the cauda epididymis and scooping it up by a round-pointed Pasteur pipette.

[0019] The chemical induction of acrosome reaction is exemplified by a method for treating fresh rat's spermatozoa with a calcium ionophore such as A23187. In addition, a method for treatment with an anionic surfactant, e.g., sodium deoxycholate, or a cationic, nonionic or ampholytic surfactant may be employed.

[0020] The induction of pseudo acrosome reaction may be achieved, for example, by physically destroying the acrosomal outer membrane to expose the acrosomal intima. Herein, the acrosome reaction by this way is referred to as "pseudo acrosome reaction". The pseudo acrosome reaction may be carried out, for example, by freezing fresh rat's spermatozoa at around -20°C and then thawing. Alternatively, electroporation or ultra-sonication may be utilized.

[0021] In a method for inducing acrosome reaction in culture, the method that fresh rat's spermatozoa are cultured in a medium containing calcium is exemplified. The concentration of calcium in the culture medium is preferably set in a range of 0.5 - 5 mM, particularly at 1.8 mM. As a source of calcium, calcium chloride, calcium nitrate, calcium lactate, calcium gluconate, or the like may be used. As for the culture medium to be used, for example, m-KRB, M-199, TYH and the like media are included. The TYH medium is composed of 6.976 g/L of sodium chloride, 0.356 g/L of potassium chloride, 0.251 g/L of calcium chloride dihydrate, 0.293 g/L of magnesium sulfate heptahydrate, 0.162 g/L of potassium hydrogen phosphate, 2.160 g/L of sodium hydrogen carbonate, 0.111 g/L of sodium pyruvate, 1 - 000 g/L of glucose, 4.0 g/L of bovine serum albumin, and 4.8 mL of 50% syrup of sodium lactate. Additionally, the medium may be added an antibiotic or anti-fungal agent, e. g., penicillin, streptomycin, amphotericin B, etc.

[0022] Moreover, the above-mentioned methods for inducing acrosome reaction may be used in combination. For example, a method comprising treatment with A23187 followed by freezing and thawing may be employed. Alternatively, fresh spermatozoa may be cultured in a medium containing calcium, during which culture A23187 is also added. In such case, the concentration of A23187 is preferably to add A23187 alone at a lower concentration than that inducing the acrosome reaction.

[0023] Immunization may be performed on a mammal such as mouse, rat, and the like. As a mammal, it is desirous to use an inbred animal strain to that of permanently growing cells used as a partner in cell fusion. Both of male and female animals may be used preferably at the age of, for example, 3 - 10 weeks old. The number of rat's spermatozoa used in immunization, for example, for one mouse, is preferably 1×10^3 - 1×10^{10}. The spermatozoa are preferably mixed with, for example, Freund complete adjuvant, Freund imcomplete adjuvant, aluminum adjuvant, pertussis adjuvant, endotoxins of gram-negative bacteria (Escherichia coli, Salmonella, etc.) alum precipitate, clay grains (bentonite), aluminum compounds, oils, vitamins, plant polysaccharides, and the like to form an emulsion, which is then administered to animals intraperitoneally, intravenously, subcutaneously, or intracutaneously. The immunization may be done 1 to 5 times at intervals of 1 - 3 weeks. In thus immunized animals, polyclonal antibodies to rat's acrosome reacted sperm can be obtained from their body fluid or antibody-producing cells contained in the body fluid. When the antibody titer has increased sufficiently by measurement the antibody titer of the animals, the antibodies or antibody-producing cells are collected.

[0024] In order to obtain monoclonal antibodies to rat's acrosome reacted sperm, it is necessary to make hybridomas by fusing the antibody-producing cell with a permanently growing cell. Preparation of hybridomas may be carried out as follows.

[0025] The antibody-producing cells can be obtained from spleen, lymph node, peripheral blood, etc. , and spleen is preferred. The spleen of the immunized mammalian is extirpated aseptically 2 - 5 days after the final immunization to make a suspension of spleen cells. As the permanently growing cells as partners for fusion, cells which have been known by the person skilled in the art and which have a permanently growing property can optionally be used, though myeloma cells have widely been used. It is preferable to use the permanently growing cells derived from an animal homologous to that from which the antibody-producing cells have been obtained. In case of mice, P3U1P3X63-Ag8.U1 (P3U1), P3/NS1/1-Ag4-1 (NS-1), SP2/0-Ag14 (SP-2) , P3X63Ag8 (X63), P3X63-Ag8. 653 (653), and the like may be used. As for the permanently growing cells, it is preferable to use those having a characteristic property utilizable as a marker in selection, for example, 8-azaguanine resistant cell line, hypoxanthine guanine phosphoribosyl transfe rase-lacking cell line, etc. These cell lines can be obtained from the American Type Culture Collection (ATCC).

[0026] In fusion, any one of these permanently growing cells is cultured in a growth medium, then washed with, for example, a DMEM medium, and collected by centrifugation prior to fusion. The fusion is achieved by mixing an antibody-producing cell with the permanently growing cell in a culture medium such as MEM, DMEM or RPMI1640, to which is added a cell-fusing agent such as polyethylene glycol. If required, a small quantity of dimethylsulfoxide may be added in order to accelerate the cell fusion. Thus resulting hybridoma is cultured in a FCS-containing MEM or RPMI1640 medium containing hypoxanthine, aminopterin, thymidine, etc. After lapse of about 1 week, the culture medium is changed to an FCS-containing MEM or RPMI1640 medium containing hypoxanthine and thymidine.

[0027] The hybridoma is then subjected to screening and cloning. In screening, the supernatant of hybridoma

culture is collected and screened by a known labeled antibody method, for example, radioimmunoassay, enzyme antibody method, fluorescent antibody method, etc., using rat's acrosome reacted sperm as antigen. Subsequently, a population of hybridomas producing a single monoclonal antibody is selected by cloning by a known technique, for example, limiting dilution or soft agar method. Screening and cloning are desirable to be repeated twice or more.

[0028] The hybridoma obtained as described above may be cultured in vitro (in a culture vessel or in a nutrition medium) or in vivo (in the living body or in animal tissue) to produce a monoclonal antibody. When cultured in vitro, the hybridoma is cultured in a suitable medium such as FCS-containing MEM medium, RPMI1640 medium, etc., and the desired monoclonal antibody can be obtained from the supernatant of the culture. When cultured in vivo, the respective hybridomas are transplanted in the abdominal cavity of an animal that has a homologous histocompatibility to an animal from which the permanently growing cell is derived, and are proliferated.

Alternatively, the respective hybridomas are transplanted in nude mice, and the monoclonal antibodies produced in the ascites may be collected. Prior to transplantation of the hybridoma, it is preferred to administer intraperitoneally a mineral oil such as 2, 6,10,14-tetramethylpentadecane (pristane).

[0029] The monoclonal antibodies contained in the supernatant of culture or the ascites may be purified according to a conventional way known by a person skilled in the art, though the antibodies can be used without purification according to the purpose. The purification may be achieved, for example, by means of salting out such as ammonium sulfate precipitation, gel filtration with Sephadex, etc., dialysis with 0.02M phosphate buffer (pH 7.2), etc., ionexchange chromatography, electrophoresis, ultrafiltration, affinity chromatography, high performance liquid chromatography, and the like.

[0030] In an embodiment of the invention, the antibodies of the invention can be used in immunoassays such as immune staining, immune precipitation, immunoblotting, etc., for example, competitive or non-competitive immunoassay, radioimmunoassay, ELISA, latex aggregation, affinity column, and the like. When ELISA is employed, a sandwich assay is preferred. Herein, the immunoassay includes all methods utilizing immune reaction such as immunohistological examination, immunoblotting, immune precipitation, and the like. The antibodies of the invention can be used in collection and purification of rat's acrosome reacted sperm since they can be utilized in the above-mentioned methods.

[0031] The antibodies of the invention can be used, for example, in evaluation of fertility of rat's spermatozoa in the reproductive and developmental toxicity studies relating to the safety study. In this method, spermatozoa to be evaluated are allowed to contact with a labeled antibody of the invention, and the label attached to the spermatozoa is detected by a way of label detection. Alternatively, the spermatozoa are allowed to contact with an unlabeled antibody of the invention, which is then allowed to contact with a labeled second antibody (antibody capable of binding to the antibody of the invention). Thus, the label attached to the spermatozoa is detected by means of label detection.

[0032] Labeling of antibodies may be effected, for example, with a fluorescent material such as FITC (fluorescein isothio-cyanate) and RITC (tetramethylrhodamine isothiocyanate), radioactive material such as radioactive iodine, radiocarbon, tritium, radium, strontium, etc., enzyme such as peroxidase, enzyme substrate, coenzyme, enzyme precursor, apoenzyme, pigment, chemilumine scent compound, luminescent material, color-producing material, magnetic material, metal particles, and the like. Production of the second antibodies and attachment of a label to the antibodies may be achieved by a conventional way known by a person skilled in the art. A method for determining a fluorescent material includes, for example, a method of observing fluorescence excited with ultraviolet ray under a fluorescence microscope, a method of quantitatively determining fluorescence intensity under irradiation of a certain excitation light, or the like. As a method for measuring radioactive materials, for example, the amount of radiation can be measured using an α-ray spectrometer, scintillation counter, ionization box, counter, etc., to determine the rat ' s acrosome reacted sperm. As a method for measuring an activity of peroxidase, for example, color development is made with o-phenylenediamine as a substrate, of which the optical density is measured to determine the rat's acrosome reacted sperm.

[0033] As for a composition for measuring fertility using an antibody of the invention, a composition in which the present antibody is kept, for example, on beads (granules), is available. The beads on which the antibody is kept include carriers such as glass, Agarose, Sepharose, Agarose-loaded porous diatom earth, hydrophilic copolymeric acryl gel, polystyrene, and the like. Preferably, superparamagnetic carriers in which a magnetizable material (e.g., Fe_2O_3) is contained in the core are used. The shape of the granules is optional, including spherical form, amorphous fractured form, etc., and a spherical form is preferred. There is no limitation in particle size, which may be, for example, in a range of several to several hundred micrometers.

[0034] Alternatively, it is also possible to examine fertility of rat's spermatozoa, for example, by observing aggregation formed by binding of the spermatozoa to the above-mentioned antibody-carrying beads. Theoretically, the beads are cocultured with a certain number of fresh spermatozoa in wells on a microplate for a certain period of time. After incubation of a certain period, the acrosome reaction starts to occur and induce binding of spermatozoa and beads due to antigen-antibody reaction. Further incubation leads to increase the number of spermatozoa binded to beads due to increase the

number of spermatozoa after induction of acrosome reaction. And the beads aggregate due to motion of the binding spermatozoa. When a certain number of spermatozoa is cocultured together with a certain number of beads, ability of inducing the acrosome reaction can be estimated from the amount of spermatozoa binding to beads. Though the state of binding of spermatozoa to beads may be observed at any time, it may be effected twice, 1 - 2 hours after incubation and 6 - 8 hours after incubation. Thus, the rate of induction of the acrosome reaction can be decided from the value after 1 - 2 hours. The cumulative number of spermatozoa in which the acrosome reaction has occurred can be decided from the value after 6 - 8 hours. In this state, when spermatozoa having fertility are present at a certain number or more, multivalent binding between granules and spermatozoa is induced and granules start to form aggregate soon, and granules or granule mass having no spermatozoa binding may disappear. Thus, it is possible to find the rate of spermatozoa having fertility by examining the spermatozoa concentration at which a certain number of beads can form aggregate.

[0035] Alternatively, the complex of spermatozoa and the above antibody-carrying beads (beads carrying a primary antibody) is gathered with a magnet to wash, and then a secondary antibody labeled with a fluorescence is added to determine the number of the rat's acrosome reacted sperm. The secondary antibodies may be those recognizing rat's spermatozoa or the rat's spermatozoa after the acrosome reaction.

[0036] When the secondary antibody recognizes rat's acrosome reacted sperm, it is desirous that the epitope recognized by the secondary antibody is different from that recognized by the primary one.

[0037] In order to practice the above test, it is convenient to make a kit comprising materials necessary for carrying out the test. Such a kit may contain granules for test, a plate having wells, a culture medium for incubating sepermatozoa and the like. The culture medium may contain mineral salts, organic acid salts, sugars, serum albumin, antibiotics, indicators, and the like. In addition, the kit may contain test tubes, tubes for centrifugation, other similar glass vessels, pipettes or similar suction instruments, a microscope, and the like. In place of the above granules for test, a kit may be included a good combination of the antibodies and the solid granules as raw material.

[0038] The plate equipped with wells using in the invention may be made of plastic, ceramics, glass, enamel, and the like. The bottom of the well may be in any shape such as flat, U- or V-shape, and the flat bottom is preferred. The vertical section of the well is usually in a circular form. The number of the wells present on a plate is optional, for example, including 96 wells. Observation is usually carried out under a microscope, preferably at a magnification of 100 - 400.

[0039] More specifically, for example, a TYH culture broth containing no sodium hydrogen carbonate and bovine serum albumin, 2-15% sodium hydrogen carbonate aqueous solution, lyophilized bovine serum albumin, FARS-91 or FARS-92 antibody-binding beads, and 20 - 70% glycerin solution are used. First, 1 mL of 7% sodium hydrogen carbonate solution is preferably added to 22 mL of TYH culture broth and mixed. Thereafter, lyophilized bovine serum albumin is added thereto and dissolved without forming foam to prepare 0.4% bovine serum albumin-containing TYH culture broth. A vial containing the culture broth, of which the mouth was capped with a flame-sterilized aluminum foil, was equilibrated in a 5% carbon dioxide gas incubator at 37°C for 1 - 18 hours, and may be used in a test. In addition, FARS-91 antibody or ETARS-92 antibody-binding beads are mixed well in a test tube mixer for about 30 seconds, of which 20 μL is immediately distributed into 0.5 mL sterilized microtube, and accurately 380 μL of the culture broth is added thereto. At this time, the concentration of the beads may be adjusted at 1.0×10^3 - 1.0×10^{10} particles/mL, preferably, 0.5×10^6 particles/mL (bead suspension) . After adjustment, the mixture is preferably preserved in a cold place. 50% Glycerin solution may be added to a spermatozoa suspension in an amount equal to that of the spermatozoa suspension at the time of counting spermatozoa in order to ease the counting.

[0040] Next, a test tube containing the culture broth is provided, to which is added fresh rat's spermatozoa. The mixture is then gently dispersed for several minutes, and the spermatozoa concentration is measured. It is preferable to prepare (spermatozoa suspension) at a concentration of which the spermatozoa number is 1×10^6 particies/mL, using the culture broth. At this time, 50% glycerin solution may be added to a spermatozoa suspension in order to ease the counting.

[0041] Next, a sterilized 96-well microplate is provided. The test can be a chieved using 4 wells of the plate. That is, 100μL of the culture broth is added to the 2nd, 3rd and 4th wells, and the prepared spermatozoa suspension is added to the 1st and 2nd wells. The 2nd well is agitated by means of pipetting, of which 100μL is added to the 3rd well. This same operation is repeated to the 4th well, from which 100μL is removed. By means of this operation, 1-, 2-, 4-and 8-fold serial dilution can be made for the spermatozoa suspension. The 1st well corresponds to 1-fold dilution, the 2nd to 2-fold, the 3rd to 4-fold, and the 4th to 8-fold. The bead suspension is suspended on a mixer, of which 100μL each is added to each well. Immediately, each well is gently agitated with the tip of pipette in sequence from the 4 th well to the 1st well. The mixture is then incubated on standing at 37°C in 5% carbon dioxide gas. Then, the aggregate in the well is observed to judge fertility.

[0042] ELISA (double layer method) may be carried out, for example, as follows. For example, rat's spermatozoa which have been incubated for a period of time sufficient to induce acrosome reaction are immobilized in the bottom of well of a plastic plate, to which is added an antibody (primary antibody) specific to the rat's acro-

some reacted sperm. Immobilization in well of the spermatozoa to be tested may be achieved, for example, by immobilization with paraf ormaldehyde, etc., and subsequently drying, for example, at 37°C for about 24 hours. If there are the rat's spermatozoa in which the acrosome reaction has occurred, an antibody will bind to them. Subsequently, after washing of the wells, for example, an enzyme-labeled second antibody is added to bind to the primary antibody. This enzyme is allowed to react with a coloring substrate, and the absorbance is measured to determine the number of the rat's acrosome reacted sperm.

[0043] ELISA (sandwich method) may be carried out, for example, as follows. An antibody (primary antibody) specific to the rat's acrosome reacted sperm is immobilized in the bottom of well of a plastic plate, to which are added rat's spermatozoa which have been incubated for a period of time sufficient to induce acrosome reaction. If there are the rat's spermatozoa in which the acrosome reaction has occurred, an antibody will bind to them. Subsequently, after washing of the wells, for example, a fluore scence-labeled second antibody is added to bind to the rat's acrosome reacted sperm. The fluorescence intensity for this fluorescent substance is measured to determine the number of the rat's acrosome reacted sperm. In this connection, the primary antibody and the second antibody may be used in a combination of either of monoclonal antibody or polyclonal antibody. However, in a combination of a monoclonal antibody and another monoclonal antibody, it is preferable that the epitope of each antibody is different each other.

[0044] As a fluorescent antibody technique, for example, a method for directly labeling with a fluorescent substance an antibody to the rat's acrosome reacted sperm (direct method), a method for labeling an antibody (second antibody) recognizing the antibody to the rat's acrosome reacted sperm (indirect method), and a method for binding a second fluorescence-labeled antibody through a complement, are included.

[0045] In a specific example of the indirect method, fresh rat's spermatozoa are diluted with 0.1% - 5.0%, preferably 0.4% - 2.0% BSA-containing TYH culture broth to prepare a mixture at concentration of 1.0×10^3 sperms/mL - 1.0×10^{10} sperms/mL, preferably 0.2×10^6 sperms/mL - 1.5×10^6 sperms/mL. Next, the mixture is incubated for a period of time sufficient for inducing the acrosome reaction, then added an antibody to the rat's acrosome reacted sperm, and allowed to react at 30 - 40°C for 5 - 60 minutes. The reaction mixture is washed with the culture broth by centrifugation, added an FITC-labeled anti-mouse IgG, and allowed to react at 30 - 40°C for 5 - 60 minutes. After the reaction completion, the mixture is washed several times with 0.001 - 0.5% NaN_3-containing PBS (Ca, Mg free) by centrifugation, and observed under a fluorescence microscope. Thus, the rate of spermatozoa for which fluorescence is recognized at the head can be determined.

[0046] The rat ' s spermatozoa used as specimen may be in a state of seminal fluid without processing such as purification or in a state of live spermatozoa isolated by swimming-up, or in a state elevated the reactivity with an antibody induced by addition of an agent such as protease to seminal fluid (spermatozoa) . The specimen may be in a state of suspending in the culture broth, or in a state of fixed or immobilized in a plate or dish made of glass or plastic, or in a state of fixation with paraformaldehyde. Fixation with para-formaldehyde may preferably be carried out at a concentration of 0.5 - 15%, preferably 3% - 10%.

[0047] This antibody can be used in an in vivo screening test of substances possibly influencing on fertility of rat's spermatozoa, for example, drugs, environmental hormones, etc. The screening test may be carried out, for example, as follows. For example, a substance to be tested is administered, e.g., orally, intravenously, intraarterially, subcutaneously, intramuscularly, intraperitoneally or applied locally (to skin, as eye drops or ear drops, nebula, suppositories, etc.) to a matured male rat of age in a week once a day for a sufficient period required for the whole process of spermatogenesis and maturation of spermatozoa. Then, the spermatozoa are collected, for example, from the cauda epididymis. The collected spermatozoa are incubated, for example, for a sufficient period required for the acrosome reaction, and their fertility is determined by an indirect fluorescent antibody method.

[0048] This antibody can also be used in an in vitro screening test of substances possibly influencing on fertility of rat's spermatozoa. The screening test may be carried out, for example, as follows. For example, a substance to be tested such as drug is added to the spermatozoa collected, for example from the cauda epididymis on a fully matured male rat of age in a week, and incubated. Then, after incubation for several minutes to several hours, for example, the fertility is determined by an indirect fluorescent antibody method.

Examples

[0049] The following examples serve to illustrate the invention for further understanding, but they should not be interpreted to limit the technical scope of the invention.

Example 1 (Method for collecting immunogens)

[0050] Wistar male rats (age in 14 weeks) were killed by exsanguination under anesthesia with ether, from which the cauda epididymides were removed and immediately placed in a Petri dish containing 10 mL of m-KRB (modified Krebs-Ringer bicarbonate medium) containing 1% BSA (bovine serum albumin; Bayer) warmed at 37°C (1 plate per cauda epididymis). Cleavages were made at 2 - 3 parts of the cauda epididymis, which was gently stirred and allowed to stand for about 5 minutes to release spermatozoa. The spermatozoa

suspension is moved into a 50 mL tube, into which a calcium ionophore A23187 (Sigma) was added thereto at the final concentration of 10μM and incubated for about 15 hours in a CO_2 incubator. After termination of the incubation, the treated spermatozoa were washed twice with PBS (-), then adjusted at a predetermined concentration, and preserved at -20°C.

Example 2 (Immunization of mice)

[0051] In Jcl femal mice (age in 6 weeks), the first day of immunization was regarded as 0 day, and booster immunization was made at the 14th, 21st and 28th days. In immunization, the above-treated rat 's spermatozoa were administered subcutaneously on the back at a dose of about 1×10^7 per shot as an equal mixture with Freund's complete adjuvant and as an equal mixture of Freund's incomplete adjuvant, respectively at the 0 day and 14th day. At the 21st and 28th days, only spermatozoa were administered intraperitoneally.

Example 3 (Method for preparation of hybridoma)

[0052] Three days after the final immunization, the spleen was taken out from the immunized mice and used in cell fusion.

[0053] A suspension of splenic cells prepared on an RPMI1640 medium (GIBCO) was mixed with SP-2 cells (murine myeloma cells) at a ratio of about 4 : 1, then permitted cell-fusion using polyethylene glycol 2000 (Sigma) and washed with an RPMI1640 medium. Thereafter, the number of cells was adjusted to be about 2×10^6 cells/mL by dilution with an RPMI1640 medium containing 15% FCS (fetal calf serum; Mitsubishi Chemical), of which 100 μL each was distributed in each well of 96-well microplates , 6 plates per mouse. Next day, 100 μL of HAT medium (hypoxanthine, aminopterin, thymidine, 15% FCS-containing RPMI1 640 medium) was added to each well. At the 2nd, 3rd, 5th and 8th days, half of the medium was replaced with a HAT medium, and at the 10th and 13th days half of the medium was replaced with an HT medium (hypoxanthine, thymidine, 15% FCS-containing RPMI1640 medium).

Example 4 (Screening by ELISA)

[0054] After treatment with A23187, the frozen-thawed spermatozoa were prepared to 1×10^6 cells/mL, of which 50 μL each was distributed in each well of a 96-well flat-bottom microplate and dried at 37°C for about 24 hours for immobilization of the spermatozoa. After immobilization, the plate was blocked with 250 μL of 2% BSA-crontaining PBS(-), sealed and preserved at 4°C before use. This was used within 5 days after preparation.

[0055] The blocking solution in sperm-immobilized plates was wasted, and 50 μL each of the supernatant taken from each well of negative control (20-fold diluted normal murine serum) and positive control (1000-fold diluted spermatozoa-immunized murine serum) as well as 50 μL of the supernatant of cell culture taken from each well 13 days after incubation was distributed in each well and allowed to react at 37°C for 2 hours. After the reaction completion, the plates were washed twice with 0.05% Tween 20-containing PBS(-), added 50 μL/well of peroxidase-labeled anti-mouse IgG (TAGO) which was one hundred thousand fold-diluted, and allowed to react at room temperature for 2 hours. The plates were then washed in the same manner, added 100 μL/well of a substrate solution (60 mg/dL of o-phenylenediamine solution, added 0. 006% H_2O_2), and allowed to react under shading at 37 °C for about 20 minutes. Thereafter, the reaction was terminated with addition of 0.5M sulfuric acid (50 μL/well), and absorbance was measured with a microplate reader (490nm-650nm). The specimen exhibiting the absorbance equal to or over that of the positive control was judged positive.

Example 5 (Screening by an indirect fluorescent antibody method)

[0056] After treatment with A23187, the frozen-thawed spermatozoa were prepared to 1×10^6 cells/mL, to 20 μL of which was added 50 μL each of negative control (20-fold diluted normal murine serum) and positive control (1000-fold diluted spermatozoa-immunized murine serum) as well as 50 μL of the supernatant of cell culture taken from each well which was positive in screening by the above ELISAS at the 14th day after incubation, and the mixture was gently stirred and allowed to react at 4°C overnight or at 37°C for 2 hours. After the reaction completion, the mixture was washed twice with 0.02% NaN_3-containing PBS (-), and 20 μL of 400-fold diluted FITC-labeled anti-mouse IgG was added, gently stirred, and allowed to react at 37°C for 1 hour. After the reaction completion, the mixture was washed in the same manner, and observed under a fluorescence microscope. The presence of fluorescence recognized at the head of spermatozoon was judged positive.

Example 6 (Cloning of fused cells)

[0057] Cloning was performed by means of limiting dilution.

[0058] The cells of the positive wells were prepared to a cell concentration of 5 - 100 cells/mL with a cloning medium (HT medium containing 10% brei clone ®), of which 100 μL each was distributed to each well of a 96-well microplate. The cloning medium (100 μL) was then immediately added to each well.

[0059] For the cells after cloning, the culture medium was changed at a rate of 2 - 3 times per week (the half was replaced with a fresh cloning medium) , and screening was performed in conformity with growth of the cells. Repetition of screening and cloning established hybrid-

omas (FARS-91 and FARS-92).

[0060] (Hereinafter, a monoclonal antibody produced by a mouse-mouse fused cell FARS-91 is referred to as FARS-91 antibody, and a monoclonal antibody produced by a mouse-mouse fused cell FARS-92 as FARS-92 antibody)

Example 7 (Determination of subclass of FARS-91 and FARS-92 antibodies)

[0061] Using a mouse monoclonal antibody isotyping kit (Amersharn), the sub-class of antibodies was identified.

[0062] Three mL of ascites fluid containing FARS-91 antibody or FARS-92 antibody was allowed to react with a typing stick at room temperature for 15 minutes, and then the typing stick was washed 3 times with 5 mL of Tris buffer saline. Then, 3 mL of peroeidase-labeled anti-mouse antibody was added, allowed to react at room temperature for 15 minutes, and washed in the same manner. Then, 3 mL of a substrate solution was added, allowed to react at room temperature for 15 minutes, and typing stick was washed 3 times with distilled water. After drying, the sub-class of the antibody was identified by a sign observed on the stick.

[0063] As results, it was confirmed that the sub-class of FARS-91 antibody is IgG_1 and that of FARS-92 antibody IgG_{2b}. Example 8 (Reactivity of FARS-91 and FARS-92 antibodies to acrosome reacted sperm)

[0064] As described above, fertility of spermatozoa can be determined by confirming whether the acrosome reaction has occurred in the spermatozoa. Accordingly, it was examined whether the monoclonal antibodies obtained in Example 6 can be utilized in the above-mentioned confirmation.

[0065] It has been reported that treatment of fresh spermatozoa with a calcium ionophore A23187 causes an acrosome reaction in mice (Lynn R. Fraser; Journal of Andrology, 3, 412-419, 1982) and humans (Green et al.; Journal of Cell Science, 32, 321, 1978).

[0066] Therefore, reactivity of FARS-91 antibody and FARS-92 antibody to spermatozoa treated with A23187 (acrosome reacted sperm) and to fresh spermatozoa in rats was examined by an indirect fluorescent antibody method.

[0067] Spermatozoa collected from the cauda epididymis of Jcl:SD male rats were prepared to 1×10^6/mL with 0.4% BSA-containing TYH culture broth. A23187 dissolved in DMSO was added so as to be the final concentration of 2 μM, and the mixture was allowed to react at 37°C for 20 minutes. As a control, DMSO used as a solvent for A23187 was added and allowed to react in the same manner. After the reaction completion, the ascites fluid containing FARS-91 antibody or FARS-92 antibody was added, and allowed to react at 37°C for 30 minutes. The mixture was washed twice with 0.4% BSA-containing TYH culture broth by centrifugation (37°C, 2800 rpm, 5 min), added FITC-labeled anti-mouse IgG, and allowed to react at 37°C for 60 minutes. After the reaction completion, the mixture was washed twice with 0.02% NaN_3-containing PBS(-) by centrifugation (room temperature, 2800 rpm, 5 min), and observed under a fluorescence microscope to measure the rate of spermatozoa exhibiting fluorescence on their head.

[0068] As results, it was found that 65.5±3.7% of spermatozoa treated with A23187 (Figs. 2A and B) reacted with FARS-91 antibody (in control spermatozoa (Figs. 1A and B) , 12.5±3.1%), while 59.8±3.8% of spermatozoa treated with A2318 7 (Figs. 4A and B) reacted with FARS-92 antibody (in control spermatozoa (Figs. 3A and B), 8.5±1.7%). From these results, it was confirmed that the FARS-91 antibody and FARS-92 antibody react specifically with spermatozoa in which the acrosome reaction has occurred.

Example 9 (Observation of frozen-thawed spermatozoa by a FITC-PSA staining method)

[0069] It has been said that freezing and thawing of spermatozoa physically destructs the plasma membrane and/or acrosomal outer membrane to lead to a state after occurrence of the acrosome reaction (hereinafter, referred to as pseudo-acrosome reaction) . Therefore, using a fluorescent pigment FITC-PSA which has specificity to the contents of acrosomal granules existing in the acrosome before acrosome reaction, it was confirmed whether frozen-thawed spermatozoa have caused the pseudo-acrosome reaction. In general, in spermatozoa in which no acrosome reaction occurs, fluorescence is observed at the sperm head since the fluorescent pigment FITC-PSA is bound to the contents of acrosomal granules in the acrosome.

[0070] Smears of fresh spermatozoa and frozen-thawed spermatozoa were prepared, allowed to stand at room temperature for 2 hours or longer to dry, fixed in 95% methanol for 2 hours, and washed 3 times by immersion in distilled water for every 10 minutes. After washing, the smears were allowed to react with a FITC-PSA (50mg/mL) solution at room temperature under shading for 3 hours. After washing with distilled water, the smears were observed under a fluorescence microscope. Spermatozoa exhibiting fluorescence on their head were judged positive.

[0071] As results, fluorescence was observed on the head of fresh spermatozoa (Fig. 5A), while almost no fluorescence on that of frozen-thawed spermatozoa (Fig. 5B). That is, it was confirmed that fresh spermatozoa cause no acrosome reaction, but in frozen-thawed spermatozoa a pseudo-acromal reaction occurs.

Example 10 (Reactivity of EARS-91 and FARS-92 antibodies to frozen-thawed spermatozoa)

[0072] Since it was proven in Example 9 that in frozen-thawed spermatozoa a pseudo-acromal reaction occurs, it was examined whether FARS-91 and FARS-92

antibodies react specifically with spermatozoa that has caused pseudo-acrosome reaction, i.e., frozen-thawed spermatozoa.

[0073] To 30 μL of fresh spermatozoa or of frozen-thawed spermatozoa (1×10^6/mL) was added 50 μL of ascites fluid containing FARS-91 antibody or FARS-92 antibody. The mixture was gently stirred, allowed to react at 37°C for 30 minutes, and washed with 0.4% BSA-containing TYH culture broth by centrifugation (37 °C, 2800 rpm, 5 min) . After washing, 30 μL of 400-fold diluted FITC-labeled anti-mouse IgG was added and allowed to react at 37°C for 1 hour. The mixture was washed twice with 0.02% NaN_3-containing PBS (-) by centrifugation (room temperature, 2800 rpm, 5 min) and observed under a fluorescence microscope.

[0074] As a result, fluorescence was observed at the head of most of the frozen-thawed spermatozoa for both of the FARS-91 and FARS-92 antibodies (Fig. 6B and Fig. 7B), while the rate of fluorescent spermatozoa was greatly reduced in fresh spermatozoa (Fig. 6A and Fig. 7A). According to Examples 9 and 10, it was confirmed that the FARS-91 and FARS-92 antibodies also react specifically with spermatozoa which have caused a pseudo-acrosome reaction.

Example 11 (Reactivity of FARS-91 and FARS-92 antibodies to spermatozoa which have been frozen and thawed after treatment with A23187)

[0075] Reactivity of the FARS-91 and FARS-92 antibodies was examined for spermatozoa which have been frozen and thawed after treatment with A23187 to cause chemical and physical acrosome reactions.

[0076] Spermatozoa collected from the cauda epididymis of Jcl:SD male rats were prepared to 1×10^6/mL with 0.4% BSA-containing TYH culture broth. A23187 dissolved in DMSO was added so as to be the final concentration of 2 μM, and the mixture was allowed to react at 37 °C for 20 minutes. After the reaction completion, the treated spermatozoa were washed twice with PBS (-), prepared to a predetermined concentration, and frozen at -20°C. After thawing, the ascites fluid containing FARS-91 antibody or FARS-92 antibody was added, and allowed to react at 37°C for 30 minutes. The mixture was washed twice with 0.4% BSA-containing TYH culture broth by centrifugation (37°C, 2800 rpm, 5 min), added 30 μL of FITC-labeled anti-mouse IgG, and allowed to react at 37 °C for 60 minutes. The mixture was washed twice with 0.02% NaN_3-containing PBS(-) by centrifugation (room temperature, 2800 rpm, 5 min), and observed under a fluorescence microscope.

[0077] As a result, fluorescence was observed on the head of nearly 100% of spermatozoa which have been frozen-thawed after treatment with A23187 in the FARS-91 (Figs. 8A and B) and FARS-92 (Figs. 9A and B) antibodies.

Example 12 (Reactivity of FARS-91 and FARS-92 antibodies to human frozen-thawed spermatozoa)

[0078] It was examined by an indirect fluorescence antibody method whether the FARS-91 and FARS-92 antibodies react to human frozen-thawed spermatozoa.

[0079] To 30 μL of human frozen and thawed spermatozoa (1×10^6/mL) was added 50 μL of ascites fluid containing FARS-91 antibody or FARS-92 antibody. The mixture is allowed to react at 37°C for 30 minutes, and washed twice with 0.02% NaN_3-containing PBS(-) by centrifugation (37°C, 2800 rpm, 5 min). Then, 30 μL of 400-fold diluted FITC-labeled anti-mouse IgG was added and allowed to react at 37°C for 1 hour. The mixture was washed in the same manner and observed under a fluorescence microscope.

[0080] As results, both of the FARS-91 (Fig. 10A) and FARS-92 (Fig. 10B) antibodies did not emit fluorescence in. reaction with human frozen and thawed spermatozoa. Thus, it was shown that these antibodies are not bound to human frozen and thawed spermatozoa.

Example 13 (Preparation of a kit for characterization of fertility)

[0081] A suspension of Dynabeads M-450 sheep anti-mouse IgG (FC) was shaken well to suspend, and its necessary amount was placed in tubes for centrifugation in a clean bench and subjected to centrifugation at 4°C and 2000 rpm for 5 minutes. After removal of the supernatant, there was added a non-specific adsorption blocker, Block Ace (Snow Brand), in an amount equal to the collected Dynabeads, and the mixture was incubated under shaking at 37°C for 1 hour. After termination of incubation, the mixture was centrifuged at 4°C and 2000 rpm for 5 minutes to remove the supernatant. There was, then, added sterilized PBS(-) twice the amount of the collected Dynabeads, and the mixture was stirred and centrifuged at 4°C and 2000 rpm for 5 minutes to remove the supernatant. This operation was further repeated twice to completely remove the non-specific adsorption blocker. Finally, PBS(-) in an amount equal to the collected Dynbeads was added to the precipitate, and then added the ascites liquid containing the FARS-91 antibody or FARS-92 antibody. The mixture was mixed well and allowed to react under shaking at 37°C for 1 hour. Because dispersion of beads was insufficient under shaking due to their heavy specific gravity, the mixture was stirred well with a mixer frequently by taking out during the reaction. After the reaction completion, the mixture was centrifuged at 4°C and 2000 rpm for 5 minutes to remove the supernatant. Then, the sterilized PBS(-) in an twice amount of the collected Dynbeads was added to the precipitation. After mixing of it well, it allowed to centrifuge at 4°C and 2000 rpm for 5 minutes to remove the supernatant. This washing operation was repeated additionally 3 times to completely remove the FARS-91 or FARS-92 antibody re-

maining unchanged. Finally, PBS (-) containing 0.02% NaN_3 and 2% bovine serum albumin(BSA) 10 times the amount of the collected Dynabeads was added to the precipitate obtained. This bead suspension was mixed well, of which 20 μL was taken into microtubes, to which was added 380 μL of 2% BSA-containing PBS(-). The number of beads was prepared to be 0.5×10^6/mL by counting on a Thoma's hemocytometer. On the other hand, spermatozoa collected from the cauda epididymis of Jcl:SD male rats were prepared to be 1×10^6/mL with 0.4% BSA-containing TYH culture broth. To 10 μL of antibody-binding beads of 0.5×10^6/mL were added 100 μL of rat's fresh spermatozoa prepared to 1×10^6/mL, and the mixture was allowed to react at 37°C under 5% CO_2.

[0082] As results, in both the FARS-91 and FARS-92 antibodies, the aggregation of beads were not observed immediately after addition of the rat's spermatozoa, but it was recognized after a lapse of 6 hours.

Example 14 (Kit)

[0083] By combining the following components, a kit was prepared:

TYH solution containing no sodium hydrogen carbonate and bovine serum albumin
7% Sodium hydrogen carbonate
Bovine serum albumin
FARS-91 beads or FARS-92 beads

Industrial Applicability

[0084] The present invention provides monoclonal antibodies reacting specifically to rat's acrosome reacted sperm. Additionally, the invention provides a kit for evaluation of fertility using said antibodies, by which fertility can be evaluated conveniently within a short period of time without requiring skillful technique or troublesome operations.

Claims

1. An antibody specifically reacting to rat's acrosome reacted sperm.
2. An antibody according to Claim 1, wherein said antibody is a monoclonal antibody.
3. A hybridoma producing an antibody according to Claim 2.
4. A hybridoma of which the accession number is FERM BP-7401.
5. A hybridoma of which the accession number is FERM BP-7402.
6. A monoclonal antibody produced by a hybridoma according to Claim 4.
7. A monoclonal antibody produced by a hybridoma according to Claim 5.
8. A monoclonal antibody according to Claim 2 or 6, wherein the sub-class is classified into IgG_1.
9. A monoclonal antibody according to Claim 2 or 7, wherein the sub-class is classified into IgG_{2b}.
10. An antibody according to any one of Claims 1, 2, 6, 7, 8 and 9, which has no immune reactivity to human frozen-thawed spermatozoa.
11. A method for evaluating fertility of rat's spermatozoa, **characterized in** using an antibody according to any one of Claims 1, 2, 6, 7, 8, 9 and 10.
12. A composition for measuring fertility of rat's spermatozoa, **characterized in** using an antibody according to any one of Claims 1, 2, 6, 7, 8, 9 and 10.
13. A method for screening a material influencing on fertility, **characterized in** using an antibody according to any one of Claims 1, 2, 6, 7, 8, 9 and 10.
14. A material selected by a method for screening according to Claim 13 .
15. A kit for evaluating fertility which is used in the method according to Claim 11 or 13, **characterized in** using an antibody according to any one of Claims 1, 2, 6, 7, 8, 9 and 10 or a composition according to Claim 12.

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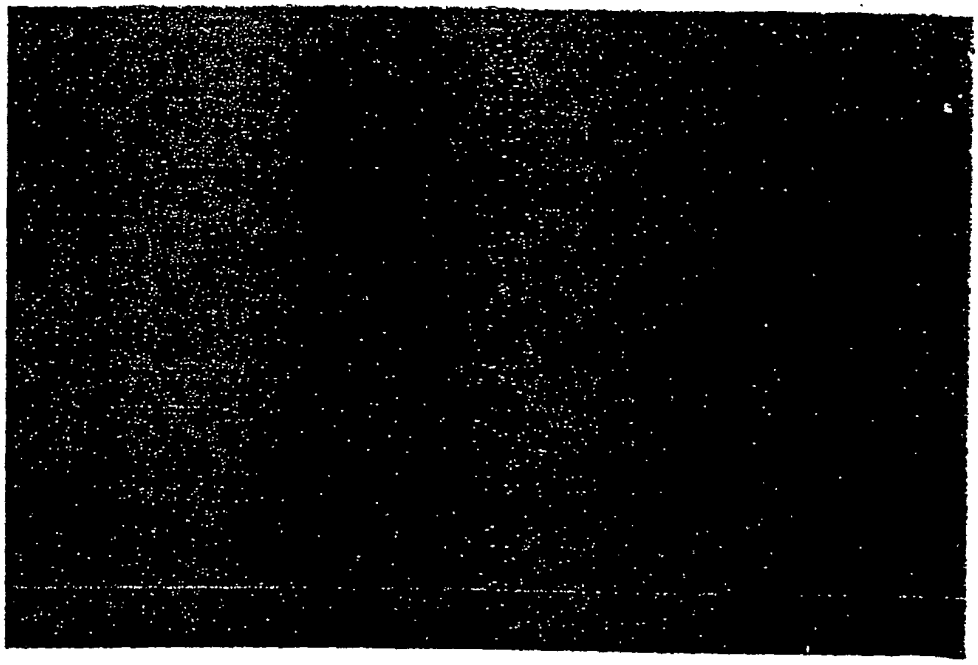

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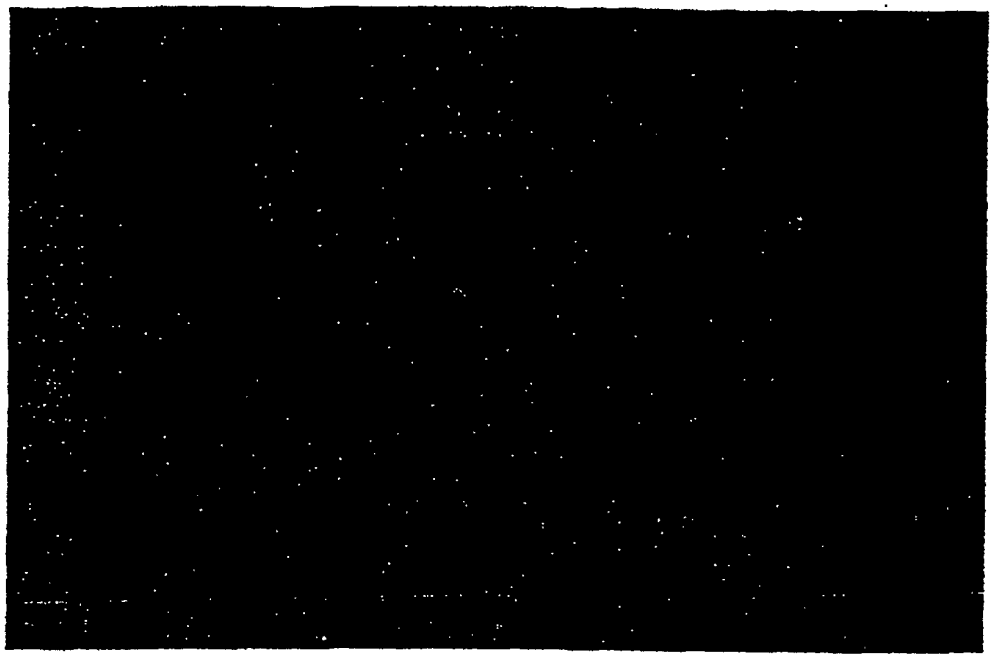

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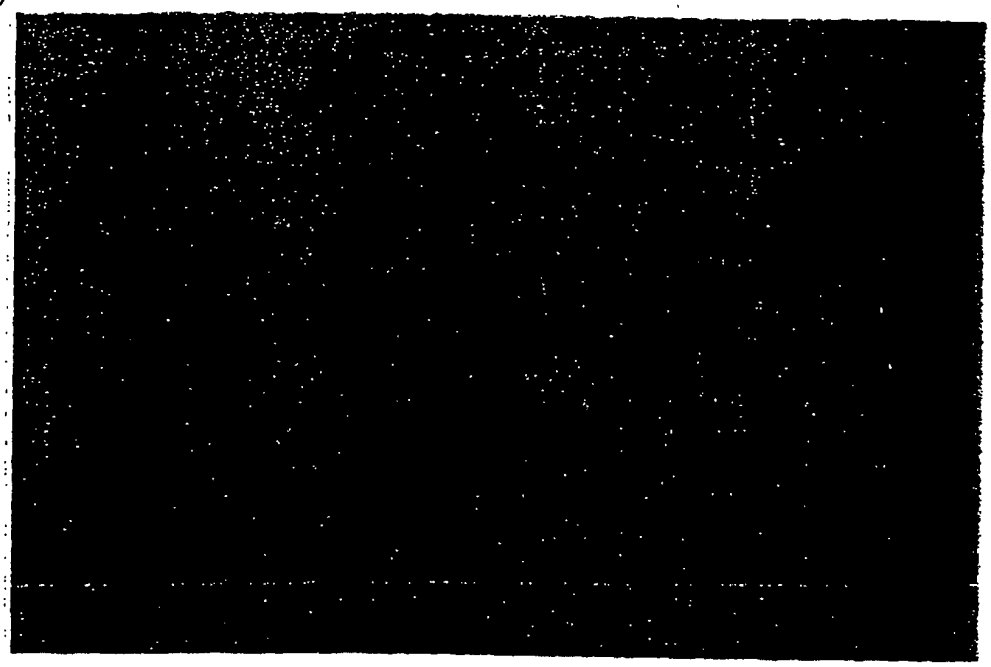

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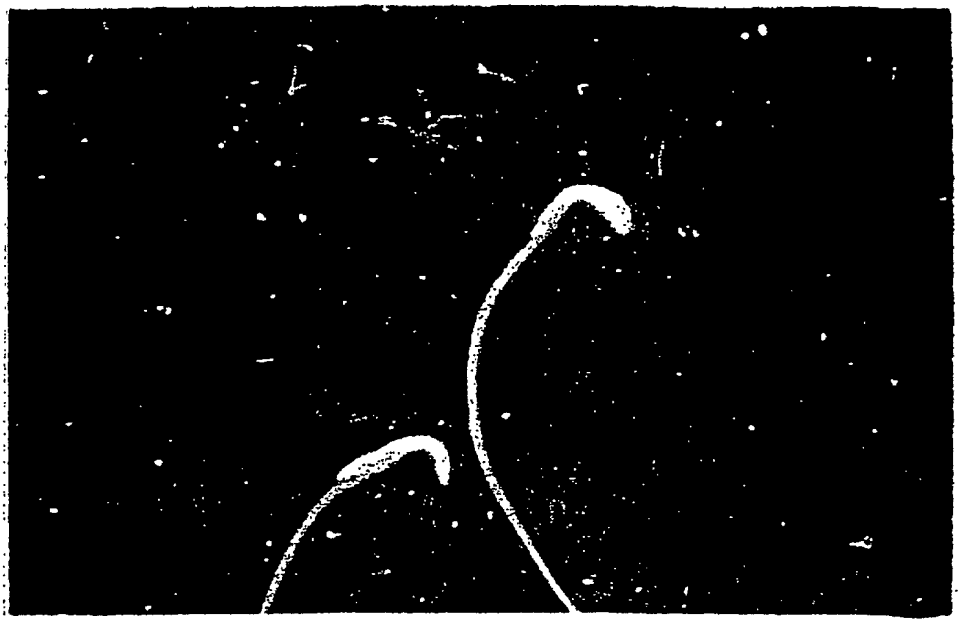

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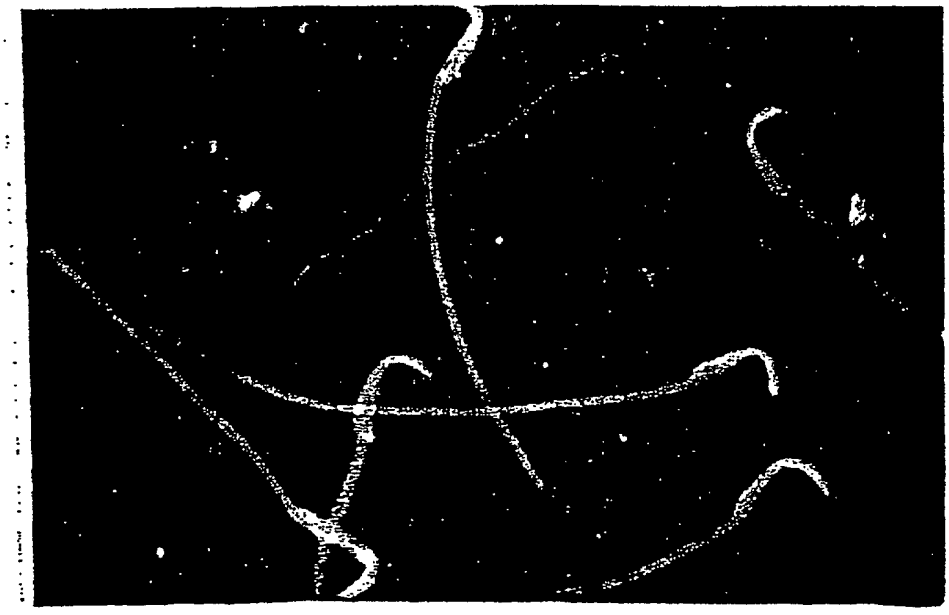

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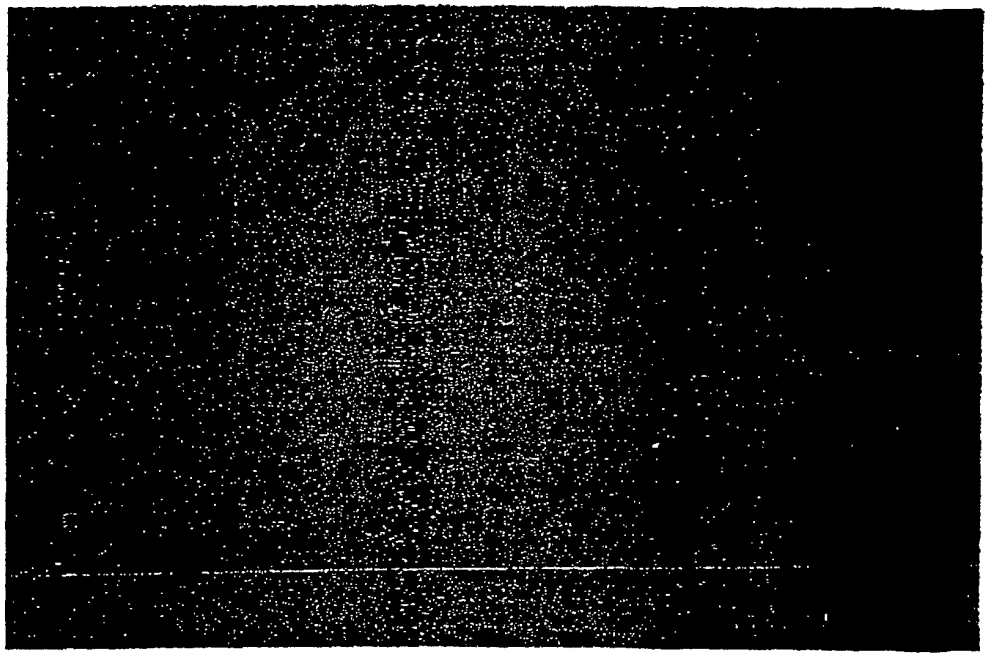

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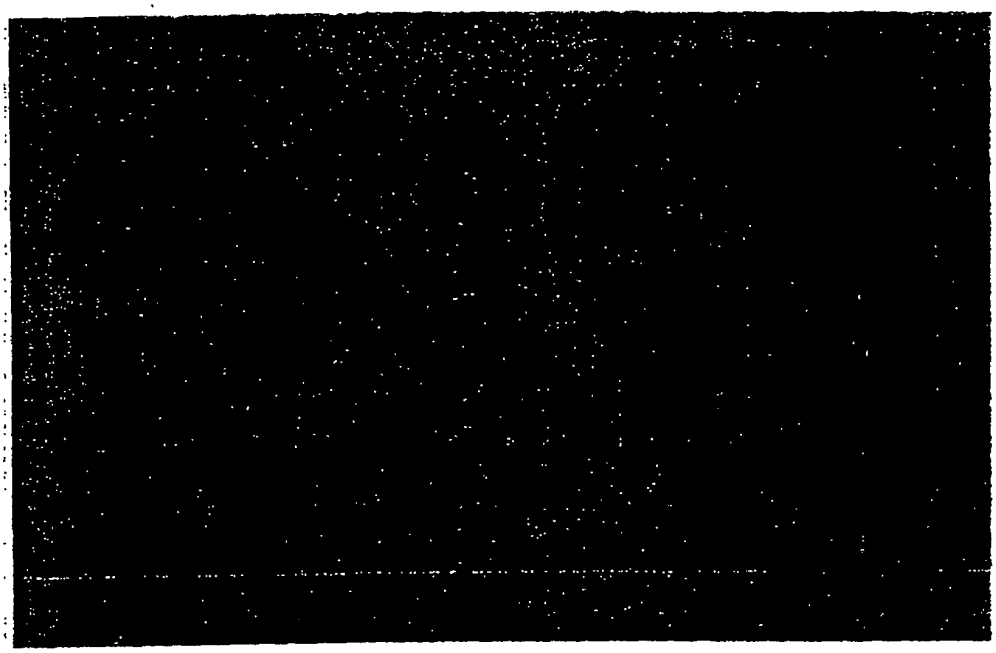

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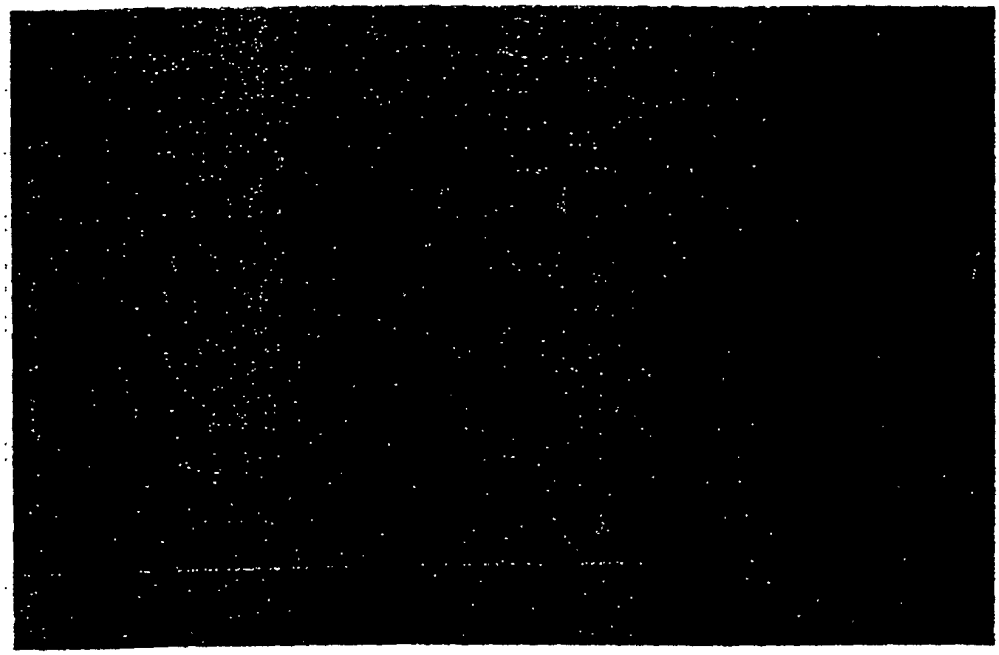
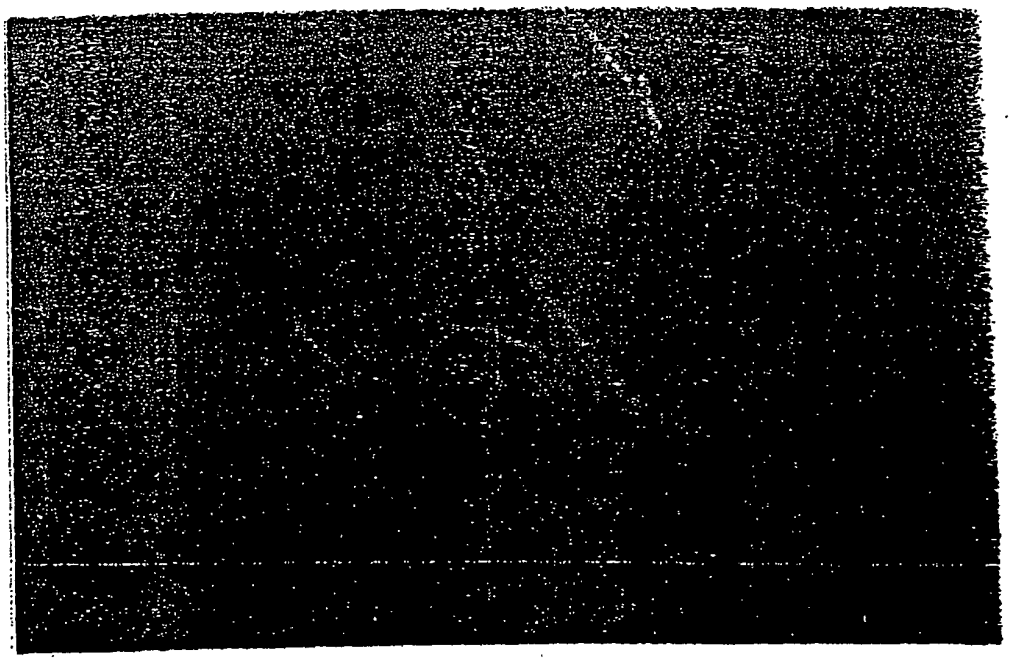
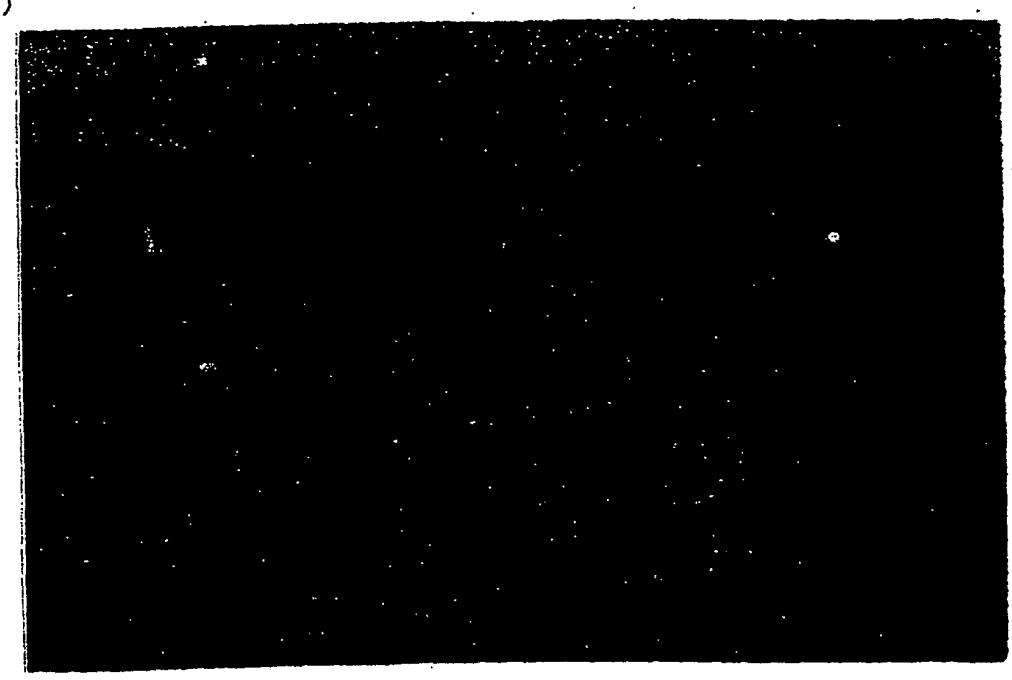


B)

A)



B)



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/07374

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C07K 16/18, C12N 5/10, G01N 33/15, G01N 33/53, G01N 33/577 // C12N 15/06, C12P 21/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C07K 16/18, C12N 5/10, G01N 33/15, G01N 33/53, G01N 33/577

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
REGISTRY(STN), CA(STN), MEDLINE(STN), WPI(DIALOG), BIOSIS(DIALOG), JICST FILE(JOIS)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OKABE, M. et al., "Capacitation-related changes in antigen distribution on mouse sperm heads and its relation to fertilization rate in vitro", J. Reprod. Immunol. (1987) Vol.11, No.2, Pp.91-100	1-3,8-15
X	YOSHIKI, T. et al., "Molecular nature of a sperm acrosomal antigen recognized by HS-13 monoclonal antibody", J. Reprod. Immunol.(1997) Vol.36, No.1-2, pp.61-75	1-3,8-15
X	JONES, R. et al., "Topographical rearrangement of a plasma membrane antigen during capacitation of rat spermatozoa in vitro", Dev. Biol.(1990) Vol.139, No.2, pp.349-362	1-3,8-15
X	SHALGI, R. et al., "Antigen on rat spermatozoa with a potential role in fertilization", Mol. Reprod. Dev.(1990) Vol.25, No.3, pp.286-296	1-3,8-15
A	EP, 387873, A (FUSO YAKUHIN KOGYO KK), 19 September, 1990 (19.09.90) & JP, 2-242697, A & AU, 9051296, A & CA, 2012264, A & JP, 3-111760, A & US, 5232834, A & DE, 69015230, E	1-15

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January, 2001 (16.01.01)	23 January, 2001 (23.01.01)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/07374

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 4-268454, A (FUSO YAKUHIN KOGYO KK), 24 September, 1992 (24.09.92) (Family: none)	1-15

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

专利名称(译)	大鼠顶体反应后抗精子的抗体及其用途		
公开(公告)号	JPWO2001030853A1	公开(公告)日	2003-05-20
申请号	JP2001533850	申请日	2000-10-23
申请(专利权)人(译)	扶桑制药工业有限公司		
[标]发明人	桑原良弘 長谷川通規 位坂清継 荒木宏昌		
发明人	桑原 良弘 長谷川 通規 位坂 清継 荒木 宏昌		
IPC分类号	C07K16/18 C12N5/10 G01N33/15 G01N33/50 G01N33/53 G01N33/577 C12N15/02 C12P21/08		
FI分类号	C07K16/18 G01N33/15.Z G01N33/50.Z G01N33/53.S G01N33/577.B C12P21/08 C12N5/00.B C12N15/00.C		
优先权	1999304530 1999-10-26 JP		
其他公开文献	JP4610149B2		

摘要(译)

顶体反应后，将对大鼠精子具有高抗体滴度的小鼠脾细胞与小鼠来源的骨髓瘤细胞融合，筛选出顶体反应后与大鼠精子强烈反应的融合细胞，并检测顶体反应后的大鼠精子。 获得了杂交瘤（FARS-91菌株和FARS-92菌株），该杂交瘤产生与该菌株特异性结合并表现出稳定生长能力的抗体。 可以从杂交瘤获得在顶体反应后选择性结合大鼠精子的单克隆抗体，并且提供了用于诊断精子繁殖力的方法来评估大鼠精子繁殖力。